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Collagen production ability of milk basic protein is dependent on stimulatory effect of transforming growth factor-β1 and β2

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ABSTRACT

Milk basic protein (MBP), a mixture of positively charged whey proteins extracted from cows' milk, regulates bone-turnover and stimulates collagen production in osteoblasts. Whey contains growth factors, including transforming growth factor (TGF)-β1, which enhances collagen production in fibroblasts and osteoblasts. However, the extent to which bovine TGF-β1 and TGF-β2 in MBP contribute to collagen production in human mesenchymal cells has not been clarified. The separated MBP fractions increased the concentration of procollagen C-terminal peptide in human dermal fibroblasts in correlation to the bovine TGF-β1 and TGF-β2 concentrations. A similar trend was observed with respect to bovine TGF-β2 and human osteoblasts. These stimulatory effects were inhibited by ALK5 inhibitor II as a TGF-β receptor I signal inhibitor. Accordingly, bovine TGF-β1 and TGF-β2 in MBP could induce collagen production in human mesenchymal lineages, such as fibroblasts and osteoblasts. These results may extend the use of MBP as a functional food ingredient.

1. Introduction

Collagen is the most abundant protein in mammals, accounting for about 30% of the total protein. To date, 28 different types of collagens have been identified. Type I collagen produced by osteoblasts and fibroblasts is the most common fibrillar collagen. It gives connective tissues, such as the bone and skin, structure and provides firmness and strength by contributing to the external structure of cells. The synthesis of type I collagen is involved in bone formation (Blair, Zaidi, & Schlesinger, 2002) and in skin development and ageing (Marcos-Garcés et al., 2014).

Milk basic protein (MBP) is a basic protein extract isolated from defatted milk by cation-exchange chromatography. MBP has regulatory effects on bone turnover, as demonstrated in both in vitro studies and animal models (Kato et al., 2000; Morita et al., 2012; Ono-Ohmachi, Ishida, Morita, Kato, & Nara, 2017; Toba et al., 2000) The major protein components of MBP are lactoferrin, lactoperoxidase, angiogenin, and cystatin C. It has been reported that these components of MBP suppress bone resorption (Cornish et al., 2004; Morita et al., 2011, 2008; Yasueda, Abe, Shiba, Kamo, & Seto, 2018). We previously demonstrated that MBP accelerates collagen production in osteoblasts (Morita et al., 2012) and fibroblasts (unpublished data); however, the active components in MBP involved in the induction of collagen production are still unclear.

A number of growth factors are found in cows' milk. Transforming growth factor (TGF)-β is the second most abundant growth factor (10–70 ng mL^{-1}), following insulin-like growth factor (IGF)-1 (5–100 ng mL^{-1}), and other growth factors are found at trace amounts (Pouliot & Gauthier, 2006). TGF-β has a wide variety of physiological activities; it controls the proliferation and differentiation of various cells, including mesenchymal cells (Eaves et al., 1991; Masui et al., 1986). Whey fractionated by cation-exchange chromatography contains growth factors, such as IGF, fibroblast growth factor (FGF), and TGF-β (Dyer et al., 2008). TGF-β1 is known to enhance collagen production in both fibroblasts (Qin, Xia, Fisher, Voorhees, & Quan, 2018) and osteoblasts (Suzuki et al., 2014) and is also involved in immunomodulation (Han, Li, Singh, Wolf, & Wang, 2012).

According to the biological effects and components of MBP, we hypothesised that TGF-β1 is involved in the stimulatory effect on collagen production in mesenchymal cells. However, the TGF-β1 content is much lower than the TGF-β2 content in milk and dairy products (Purup, Vestergaard, Pedersen, & Sejrsen, 2007),

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suggesting that in MBP, the TGF-β2 content is more than the TGF-β1 content. However, to the best of our knowledge, the stimulatory activity of bovine TGF-β2 on collagen production in human mesenchymal cells has not been determined. In this study, we investigated collagen production in fibroblasts and osteoblasts with fractions of MBP separated by chromatographic techniques. Activity was evaluated with respect to the concentrations of TGF-β1 and TGF-β2 in MBP fractions. Furthermore, the stimulatory effects of the MBP fractions on cells were investigated using a TGF-β signal inhibitor.

2. Materials and methods

2.1. Fractionation of MBP by column chromatography

MBP was prepared as described previously (Toba et al., 2000). In brief, fresh bovine milk was defatted by centrifugation and separated by cation-exchange chromatography. The adsorbed protein was eluted with 1 M sodium chloride (NaCl). The eluted fraction was desalted using a cellulose membrane tube (cut-off 14 kDa; Sanko-Junyaku, Tokyo, Japan) and lyophilised as MBP. The protein content of the MBP sample was 98% (w/w), as determined by the Kjeldahl method for nitrogen determination (conversion factor, 6.38). The solution of MBP (0.2 mg) in 10 mM sodium phosphate buffer (pH 7.0) was loaded onto a series of cation-exchange columns, including the HiTrap SP column (25 mm × 155 mm; GE Healthcare, Chicago, IL, USA) for the fibroblast assay and the SP-Sepharose column (25 mm × 155 mm; GE Healthcare) for the osteoblast assay, and equilibrated with the same buffer at a flow rate of 5 mL min^{-1}. The bound proteins were washed with buffer and eluted in an NaCl gradient in buffer as follows: 0–125 min, 0–0.6 M; 125–200 min, 0.6–1.5 M. The elution profiles were monitored by measuring absorbance at 280 nm. Fractions used to observe stimulatory effects on collagen production were pooled, concentrated using the Amicon Ultra 15-Ultracel 5k (EMD Millipore, Burlington, MA, USA), and loaded onto a HiLoad 16/60 Superdex (GE Healthcare) 75 pg column (16 mm × 600 mm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.2 mL min^{-1}. Subsequently, the protein concentration in each fraction was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

2.2. Measurement of bovine TGF-β1 and TGF-β2 in the fractions

The concentrations of bovine TGF-β1 and TGF-β2 in the fractions were determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Emax TGF-β1 and TGF-β2 ELISA Kit; Promega, Fitchburg, WI, USA). The samples were first treated with 1 M HCl and neutralised with 1.2 M NaOH before analysis by ELISA. The samples were plated in duplicates on the ELISA plate.

2.3. Culture of human dermal fibroblasts

Primary neonatal human dermal fibroblasts (HDFs) were obtained from Lifeline Cell Technology (Walkersville, MD, USA) and grown at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% foetal calf serum (FCS) and penicillin and streptomycin (100 U mL^{-1}; Life Technologies) to near-confluence (70–90%). HDFs at fewer than six passages were used for all experiments. HDFs were reconstituted with Medium 106 (Life Technologies) supplemented with 10% FCS and were inoculated into each well (8000 cells $well^{-1}$) in a 24-well plate (Becton Dickinson, Franklin Lakes, NJ, USA), which was followed by an additional 3 days of incubation to reach 100% confluence.

For the inhibition assay, ALK5 inhibitor II (Enzo Life Sciences, Farmingdale, NY, USA), a TGF-β receptor I (ALK5) signal kinase inhibitor that is non-specific for TGF-β1 and TGF-β2, was first dissolved in dimethyl sulfoxide at a concentration of 10 mg mL^{-1}. Recombinant human TGF-β1 (rhTGF-β1; Peprotech EC, London, UK) and TGF-β2 (rhTGF-β2; Peprotech EC) were used as positive controls at 20 μg mL^{-1} after activation with sterile 4 mM HCl containing 1 mg mL^{-1} BSA. After diluting substances with Medium 106 (1 and 10 μg mL^{-1} MBP, 1 μg mL^{-1} each MBP fraction, 0.2 and 2 ng mL^{-1} activated rhTGF-β1, and 500 nM ALK5 inhibitor II), all culture medium was replaced and further cultured for 3 days.

2.4. Culture of osteoblastic MG63 cells

Human osteoblastic MG63 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan), and the assay was performed as described previously (Morita et al., 2012). In brief, cells were reconstituted with DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin, inoculated into each well (2000 cells $well^{-1}$) of 96-well plates (AGC Techno Glass, Shizuoka, Japan), and cultured for 4 days. The culture medium was replaced with DMEM supplemented with 1% FCS containing samples and ALK5 inhibitor II, and the cells were cultured for 4 days.

2.5. Measurements of procollagen type I C-peptide and DNA

The cell supernatants were collected, and the amount of procollagen type I C-peptide (PICP) was determined using a PICP Enzyme Immunoassay Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The cultured cells were harvested using a cell scraper (AGC Techno Glass) after incubation with trypsin–EDTA solution (Thermo Fisher Scientific) at 37 °C for 1 min, and cellular DNA was purified using QIA Shredder (QIAGEN, Gaithersburg, MD, USA). The amounts of DNA were determined using a DNA Quantity Kit (Cosmo Bio, Tokyo, Japan). Finally, collagen production was calculated as g PICP g^{-1} DNA in each MBP fraction.

2.6. Statistical analysis

All experimental data are expressed as means ± standard deviations. Statistical analysis was performed using Stat View® ver. 5 (SAS Institute Inc., Cary, NC, USA). Differences between groups were estimated by Tukey–Kramer post-hoc tests or Dunnett's tests. Differences between two groups were analysed with an unpaired Student's *t*-test. A p-value < 0.05 was considered significant.

3. Results

3.1. Specific MBP fractions stimulated collagen production by fibroblasts

We separated MBP by column chromatography into 20 fractions (Fig. 1A). Protein was detected in fractions 1–15, while fractions 12–14 had particularly high concentrations as observed by the absorbance at 280 nm. We evaluated the ability of each MBP fraction to stimulate collagen production in HDFs. Increased PICP concentrations were observed in the supernatants of HDFs using fractions 2–3 and 8–10 (Fig. 1B). In particular, 2.0–2.4-fold higher concentrations of PICP were observed using fraction 3 and fraction 9 than those observed using fractions 6, 7, and 14. The concentrations of both TGF-β1 and TGF-β2 in the MBP fractions were determined.

Approximately 37 μg g^{-1} TGF-βs was detected in MBP, and TGF-β2 was the predominant isoform (TGF-β1:TGF-β2 = 1:4.4). TGF-β1

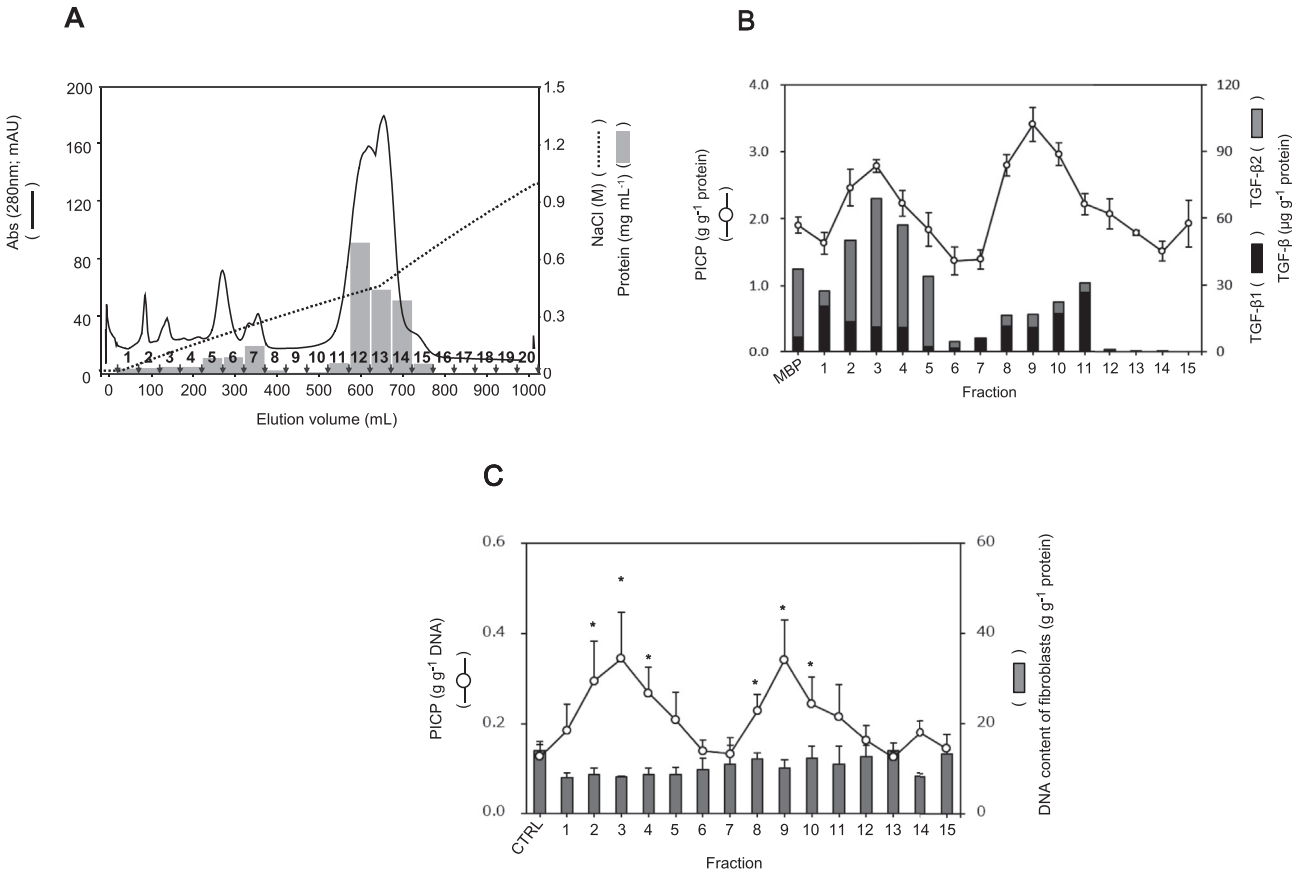

Fig. 1. Elution profile (A) of cation-exchange column chromatography of MBP (arrows and numbers represent the collection periods and fraction numbers, respectively), the stimulatory effect of MBP fractions on collagen production in fibroblasts (B) and the concentration of PICP relative to the DNA content of fibroblasts (C; values represent means ± SD (n = 3), $^{*}p < 0.05$ versus control).

and TGF-β2 had distinct elution profiles; TGF-β2 eluted earlier than TGF-β1. The DNA amounts of HDFs did not increase by the addition of MBP fractions (Fig. 1C). The PICP/DNA ratio was significantly higher in the cells supplemented with fractions 2–4 and 8–10 than in the control ($p < 0.05$, Fig. 1C). In fraction 3 and fraction 9, the PICP/DNA ratio was 2.6-fold higher than that in the control ($p < 0.05$).

3.2. TGF-β in MBP stimulates collagen production by fibroblasts

We evaluated the effects of rhTGF-β1 and rhTGF-β2 on collagen production in HDFs. The concentration of PICP in the cell cultures with rhTGF-β1 and rhTGF-β2 was significantly higher than that in the control, when the rhTGF-β1 and rhTGF-β2 concentrations were over 2 ng mL^{-1} and 0.2 ng mL^{-1} ($p < 0.01$; Fig. 2A). The elevations in

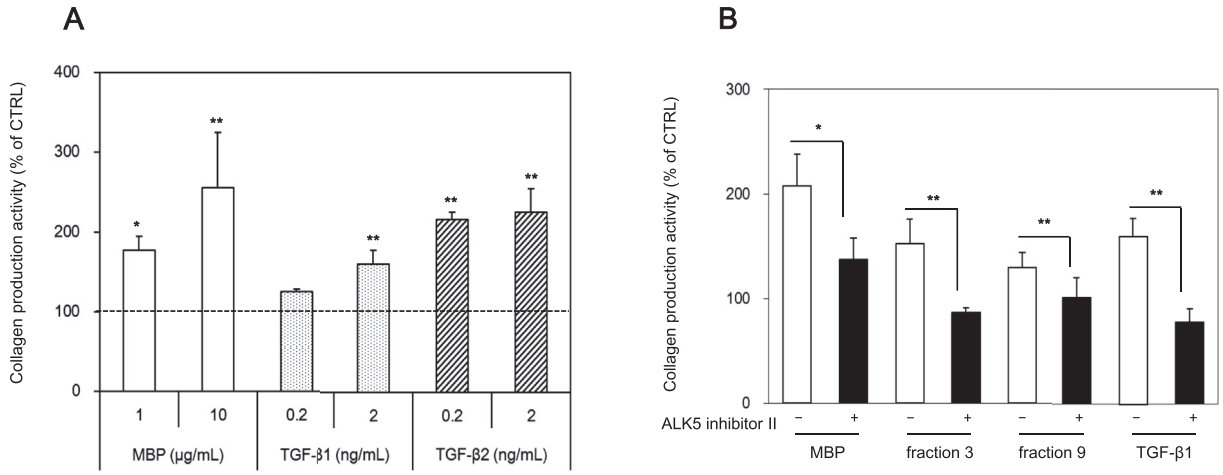

Fig. 2. Stimulatory effect (A) of MBP and TGF-β1 on collagen production by fibroblasts and (B) the suppressive effect of an ALK5 signal kinase inhibitor. Values are presented as means ± SD (n = 3); $^{*}p < 0.05$; $^{**}p < 0.01$ versus control.

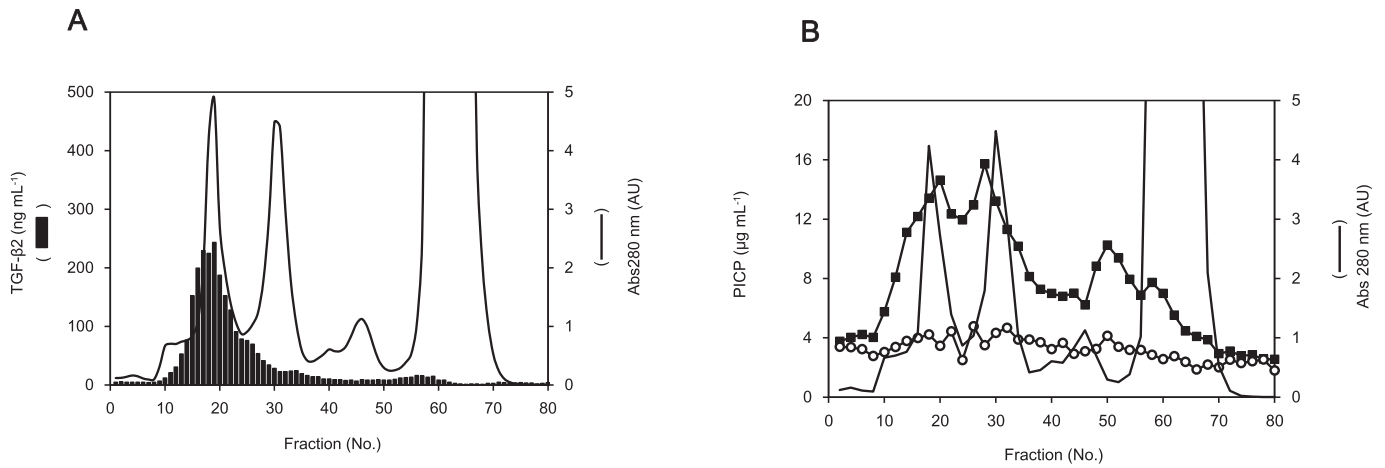

Fig. 3. TGF-β2 content in MBP fractions (A) and the stimulatory effect (B) of MBP fractions on collagen production by osteoblasts: ■, MBP fraction alone; ○, MBP fraction plus inhibitor.

the PICP concentration were significantly decreased in the supernatants of HDFs with MBP, the MBP fractions (fractions 3 and 9), and rhTGF-β1 co-cultured with ALK5 inhibitor II ($p < 0.01$, Fig. 2B). No significant decrease in the PICP concentration was observed in the control HDFs supplemented with the inhibitor only (data not shown).

3.3. TGF-β in MBP stimulates collagen production by osteoblasts

We evaluated the stimulatory effect of the MBP fractions on collagen production using the human osteoblastic cell line MG63. TGF-β2 was detected in fractions 10–60 and was relatively more abundant in fractions 14–30 from the MBP sample (Fig. 3A). The concentrations of PICP were high in MG63 cultures with chromatographically separated MBP fractions 10–60 and were particularly high (3–4-fold higher than that of the control) for fractions 14–30 (Fig. 3B). These effects were largely suppressed by the addition of ALK5 inhibitor II.

4. Discussion

In this study, we separated MBP by cation-exchange chromatography and identified components of MBP with stimulatory effects on collagen production using fibroblasts and osteoblasts. The collagen production activity of specific MBP fractions was related to the concentration of TGF-βs (β1 and β2). These results were verified using recombinant human TGF-β1 and TGF-β2 and an inhibitor of the TGF-β receptor I (ALK5) signalling pathway, through which both TGF-β1 and TGF-β2 act. The TGF-βs were abundant in MBP ($\approx 40\ \mu g\ g^{-1}$), explaining the effects on cell differentiation (collagen production). Indeed, it has been reported that TGF-β1 stimulates collagen production and differentiation in osteoblasts (Suzuki et al., 2014), but TGF-β2 is more abundant than TGF-β1 in cows' milk. The two isoforms (β1 and β2) share about 70% homology (Derynck et al., 1988), and the action of TGF-βs is mediated by a heteromeric receptor complex, composed of type I (TβRI/ALK5) and type II, at the cell surface that transduces intracellular signals via the Smad pathways or mitogen-activated protein kinase cascade (Feng & Derynck, 2005; Wu, Chen, & Li, 2016).

Few studies have examined the bioactivity of TGF-β2, which plays a role in immunomodulation, including the development of immune tolerance to prevent autoimmunity and anti-inflammatory responses (Fell et al., 2000; Oz, Ray, Chen, & McClain, 2004). This study is the first to report that bovine TGF-β2 obtained from cows' milk actually has stimulatory effects on collagen production similar to those of bovine TGF-β1, as demonstrated in a series of human mesenchymal cell lineages.

Type I procollagen has propeptide extensions at both ends of the molecule. Specific enzymes remove these propeptides before the collagen molecules are assembled into fibres. A fragment removed from the carboxy-terminus, PICP, is secreted by cells, and its concentration reflects type I collagen synthesis (Seo et al., 2017); accordingly, we employed PICP ELISA to evaluate type I collagen synthesis. In our study, TGF-βs in MBP increased the concentration of PICP in the confluent cell cultures independently of cell proliferation, consistent with previous findings (Varga, Rosenbloom, & Jimenez, 1987). We previously demonstrated that MBP increases the expression of type I collagen messenger RNA (Morita et al., 2012). Combined, these results suggest that TGF-βs in MBP increase type I procollagen synthesis. However, TGF-β1 stimulates the processing of procollagen into collagen as well as procollagen synthesis (Chua, Geiman, Keller, & Ladda, 1985). Both reactions result in an increase in the PICP concentration; therefore, further studies are required to clarify the mechanisms underlying the increased PICP production by MBP.

Other studies have suggested that orally administered TGF-β can exert effects via transfer into the systemic circulation (Ando et al., 2007; Oz et al., 2004). TGF-β forms dimers through disulphide bonds, which may partially explain the relative resistance to extreme pH conditions, such as in the presence of gastric acid (Klagsbrun, 1978). TGF-β becomes active in acidic conditions (Pakkanen, 1998) and promotes systemic effects. A previous study showed that oral administration of MBP to ovariectomised rats can offer protection against osteoporosis and increase the amount of proline, hydroxyproline, and hydroxylysine, which are amino acid components of collagen in the bone (Kato et al., 2000). Accordingly, our current results suggest that MBP can enhance bone formation by inducing collagen production via the TGF-β signalling pathway. It is of interest to examine whether TGF-β contained in orally administered MBP has a stimulatory activity on collagen production locally, even after acidification with gastric acid and subsequent digestion.

5. Conclusion

Our data support the hypothesis that the effects of MBP on collagen production in fibroblasts and osteoblasts were mediated by TGF-β signalling. Bovine TGF-β1 and TGF-β2 were related to the

observed effects. TGF-β is widely accepted as a strong, bioactive molecule.

MBP is a complex of several proteins with different properties; this suggests that MBP exerts greater effects than those of individual milk proteins. Our results may lay the foundation for further studies and help extend the use of MBP as a functional food ingredient enriched with TGF-β, in addition to other components in MBP.

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